REMARKS

I. Status of Claims

Claims 1-10, 21 and 22 are pending and stand rejected under the first paragraph of §112. Claims 1-3 stand rejected under §102 over Zong *et al.* ("Zong"). The specific grounds for rejection, and applicants' responses thereto, are set out in detail below.

II. 35 U.S.C. §112, First Paragraph

Claims 1-10, 21 and 22 continue to be rejected under the first paragraph of §112 as lacking an enabling disclosure. The examiner states that the specification is not enabling due to the fact that the claims encompass refolding of many different proteins, while the specification discloses only limited examples. Thus, it is argued that it would take undue experimentation to apply the claimed invention to other proteins. Again, applicants respectfully traverse.

A. The Understanding in the Art Permits Extrapolation From the Results of the Present Invention to a Wide Variety of Different Proteins

Although the realization that high pressure could be used to effect refolding of ***aggregated*** proteins was not made until the current invention, the general effects of pressure on protein structure are well known to those skilled in the art. For example, the closed-loop folding diagrams for native protein stability in pressure-temperature space were first described in 1971 in the pioneering work of Hawley (2). The thermodynamics and mechanisms of native structural transitions, as a function of pressure and temperature, are well described in the literature (3-39). The present invention makes the leap that the same mechanisms and thermodynamics apply to systems containing aggregated, non-native proteins. Once this conceptual leap (which is

described in the application) is disclosed, it is a relatively simple matter for those skilled in the art to make and use the invention.

The examiner also objects to the generality of the claims in covering the refolding of all proteins. However, it is submitted that the thermodynamics of pressure interactions with protein are indeed general, having been demonstrated for such widely varying types of proteins as antibodies (40-44), lysozyme (5,6,9,45), ribonuclease, DNA polymerase (22), DNA binding proteins (46), yeast glyceraldehyde 3-phosphate dehydrogenase (47), arc repressor protein(48), alpha-chymotrypsin (49), ribulose 1-5 biphosphate carboxylase oxygenase (50), glycogen phosphorylase (51), β-galactosidase (52-54), rhodanase (55), lactate dehydrogenase (56), bovine pancreatic trypsin inhibitor (57), brain enolases (58), invertase (59), phage-p22 tailspike protein (21), subtilisin (60), cholinesterase (61), glutamate dehydrogenase (62), trypsin (25), Cytochrome p-450 (63), trp repressor (26), allophycocyanin (64), topomyosin, creatine kinase (65), carboxypeptidase-Y (66), thermolysin (67), chaperonins (68), protein-membrane complexes (69), viral coat proteins (70), F1-ATP-ase (71) and triose phosphate isomerase (72). These proteins include all of the classes cited by the examiner on page 3 of the final Office Action, with the exception of RNA-containing proteins, which have not been discovered yet. Thus, there is clearly sufficient information within the present disclosure, taken in light of the knowledge of protein pressure thermodynamics, for someone skilled in the above-cited art to practice the present invention.

B. The Present Invention Provides a New Paradigm for Controlling Refolding of Aggregated Proteins

It also is well established in the published literature that moderate pressures can cause dissociation of native multimeric proteins into the constituent monomers, but that much higher

pressure is needed to unfold the monomers. As detailed in the specification (p. 13, lines 23-31 to p. 14, line 9), multimeric proteins tend to dissociate into their respective subunits at pressures in the range of 1-3 kbar, while monomeric proteins typically require pressures above 4 kbar to cause unfolding. There is, thus, a well-defined "pressure-window" described in the literature where monomeric proteins retain significant secondary structure, yet multimeric proteins are unstable.

An aggregate may be thought of as a multimeric form of a protein, albeit typically with non-native structure. In addition, non-native aggregates can have a range of molecular weights, from twice that of the native protein, to essentially infinite molecular weight for insoluble precipitates. The present invention makes the conceptual leap that the same thermodynamics and pressure-window that apply to native multimeric and monomeric proteins also govern the behavior of non-native protein aggregates under high hydrostatic pressures. Furthermore, recent mechanistic studies have documented that the dominant effect of hydrostatic pressure on non-native aggregates is to weaken hydrophobic interactions, allowing facile disaggregation. This same mechanism has been previously established for high-pressure dissociation of native multimeric proteins (73). Thus, for one skilled in the art, the pressure ranges specified in the present specification allow application of the invention to proteins in general.

In the instant application, the inventors have provided data for pressure-induced disaggregation and refolding from non-native aggregates of lysozyme (a disulfide bond-containing protein), recombinant human growth hormone (an α-helix bundle, metal binding, hormone receptor binding protein), interferon-γ (an α-helix bundle protein, hormone receptor binding protein), and β-lactamase (an enzyme). In literature appearing after the filing of the instant application, pressure-induced disaggregation and refolding has been demonstrated for p22

tailspike protein (a filament-forming protein) (21). Thus, pressure-induced disaggregation has been demonstrated for most of the protein classes cited by the examiner, and the great body of literature on high-pressure effects on proteins suggests that protein aggregates in general are amenable to this treatment.

The examiner states on pages 3 and 4 of the final Office Action that "It is well known in the art that most proteins, if denatured or aggregated in an inclusion body, do not readily renature using a single method of treatment. Some proteins, if denatured or aggregated in an inclusion body, have no treatment that can be used to yield active protein (*e.g.*, insulin and other multimeric proteins)." It is submitted, respectfully, that this statement has little bearing on the current invention. Moreover, there are numerous examples of multimeric proteins, ***including insulin***, that have been refolded from inclusion bodies, albeit using conventional (*i.e.*, expensive and time consuming) chemical methods. For a review, see (74) and references therein. A substantial patent literature (*e.g.*, U.S. Patent 5953461) for refolding of insulin exists.

In maintaining this rejection, the examiner has focused on the "common aggravation of inclusion body formation" in arguing for a significant measure of the unpredictability in the art. However, it is important to recognize that the present invention uses the inclusion body state and other improperly folded states (*e.g.*, non-native aggregates) as a starting point, rather than considering them a "dead end." By taking advantage of the fundamental thermodynamics of pressure interactions with proteins, disaggregation and refolding is used to create native, functional proteins molecules from what was once was considered to be an unrecoverable waste product.

Applicants also emphasize that the current understanding of protein aggregation provides a sound scientific basis for the general application of the present invention to protein aggregates.

Historically, research on protein aggregation first led to the proposal that protein aggregates formed from the fully unfolded, denatured state (reviewed in (75)). Much of this early work focused on thermally-induced protein aggregation, precipitation and gelation, in which it was found that incubation of a protein at a temperature above its thermal melting point fosters aggregation. However, subsequent research documented that even under these conditions, aggregates were formed from protein molecules prior to their full unfolding. The next historical insight into aggregation came when it was found that, under certain relatively destabilizing conditions (*e.g.*, pH 3.0) that grossly perturbed protein tertiary structure, secondary structure could be maintained. The protein species formed was termed "molten globule," which is often also found to be an intermediate on the protein-folding pathway (*e.g.*, (76-79)). If incubated at sufficiently high concentrations (*e.g.*, 1 mg/ml), molten globules readily form aggregates (76, 78-80).

Most recently, the inventors have found that, even under solutions conditions that are not perturbing of tertiary structure and which thermodynamically greatly favor the native state, proteins can form aggregates and precipitates (81, 82). The native conformation is a dynamic structure, such that any instant in time there exist an ensemble of species with a distribution of structural expansion/compaction. The inventors propose that these aggregates are formed from species within the native state ensemble that are structurally expanded relative to the most compact conformations (81, 82). Traditional protein refolding protocols that use high concentrations of chaotropic agents such as urea or guanidinium-HCl first completely unfold proteins, then gradually remove chaotrope (*e.g.*, by dialysis). In the traditional process, as chaotrope is removed, conditions that favor population of aggregation-prone molten globule states are encountered. Furthermore, the reduction in chaotrope concentration obviates the

capacity of these compounds to act as protein solubilizing agents. As a result of these two factors, the traditional refolding protocol is often plagued with massive re-aggregation of the protein during during removal of the chaotrope and concomitant refolding. In contrast, under the "pressure-window" conditions used in the present invention, the monomeric protein molecules produced by disaggregating non-native aggregates are thermodynamically blocked from re-aggregating, allowing them time to refold to the thermodynamically favored, compact native state.

C. The Present Application Provide Sufficient Instruction to One of Skill in the Art

The examiner states that no guidance is given in the application for the pressure ranges of the process. However, it is submitted that sufficient information for one skilled in the art of thermodynamics of proteins at high pressure (see for example, (2)) to rapidly determine optimal pressure conditions for a given protein. The preferred ranges of pressure provided in the application have proved adequate with minimal experimentation for disaggregating and refolding of several proteins, including the four proteins described in detail in the application (lysozyme, human growth hormone, interferon-γ, and β-lactamase), as well as a number of other proteins currently under study in the inventors' laboratory (granulocyte colony stimulating factor (GCSF) - a helical protein, bikunin, and aVEGF; see attached declaration), and in the labs of others (*e.g.*, rhodanase (83), and p22 tailspike protein (21)).

To summarize, there are a number of ways of classifying proteins. For example, proteins may be classified by secondary structure as a-helical, β-sheet, or α/β structures. Growth hormone, bikunin, and lysozyme, are examples of each of these three categories, respectively. Another categorization is glycosylated versus non-glycosylated. β-lactamase, granulocyte

colony stimulating factor, growth hormone, and interferon-γ are examples of the former, while aVEGF and bikunin are examples of the latter. Another categorization is disulfide bonded versus non-disulfide bonded. Lysozyme, aVEGF, and bikunin contain disulfide bonds, while growth hormone, β-lactamase, granulocyte colony stimulating factor and interferon-γ do not. Yet another classification of proteins is by quaternary structure. These include, for example, monomeric proteins, dimeric proteins, and tetrameric proteins. For multimeric proteins, the categories can be further subdivided into homo- and heteromultimers. The present inventors have shown that our invention applies to monomers (growth hormone, β-lactamase, granulocyte colony stimulating factor and bikunin), dimers (interferon-γ, a homodimer), and tetramers (aVEGF, a heterotetramer). Yet another classification is based on function, which includes reaction catalysis (lysozyme, β-lacatamase), binding to receptors (growth hormone, interferon-γ, granulocyte colony stimulating factor), binding to antigens (aVEGF), inhibitors (bikunin), and binding to metals (growth hormone). Thus, by numerous different protein classification schemes based on protein structure and function, the inventors have provided ample examples of the applicability of our invention to broad classes of proteins.

Thus, applicants submit that the present application provides a sufficient basis for finding the present claims enabled. Further, though not required, the attached declaratory submission provides additional evidence showing that one of skill in the art, using only the methods disclosed in the instant specification, could make and use the invention as now claimed. Reconsideration and withdrawal of the rejection is, therefore, respectfully requested.

III. Rejection Under 35 U.S.C. §102

Claims 1-3 are rejected as anticipated by Zong. According to the examiner, Zong discloses the use of a 2 kbar pressure step with the addition of oxidized glutathione and dithiothreitol to refold recombinant chloroperoxidase from a pellet. These actions are said to meet the limitations of the claims. Applicants traverse.

In the methods described by Zong, the starting material for high pressure treatment is not the pellet, but rather a soluble apo protein. Zong does form pellets, but does not use them in their high-pressure treatment. In the Materials and Methods section of the aforementioned paper (page, 12421, column 1), the authors state:

> The partially purified apoCPO was diluted to 100 μg/ml in refolding medium, which contained 20 mM potassium phosphate buffer, pH 6.5, 10 μg/ml bovine hemin (Sigma, Type 1), 1 mM GSSG, and 1 mM DTT. The solution was left to stand for 48 h at 4°C. ***Precipitated protein was then removed by centrifugation.*** Small aliquots of the CPO ***supernatant*** fraction obtained in the preliminary reconstitution step were transferred to a series of 100 mM buffers .... All samples were pressurized to 207 MPa in 34.5 MPa increments, and then the temperature was lowered to –12°C in 2°C steps.

Furthermore, in previous processing steps, Zong took additional care to remove aggregated protein by centrifugation before attempting to obtain the holochloroperoxidase from the apoprotein found in soluble fractions. On page 12421, column 1, the authors state that:

> This procedure gave a urea-solubilized apoCPO protein that was about 70% pure, as judged from SDS-PAGE analysis. ***After centrifugation*** at 12000 g for 10 minutes, the ***soluble fraction was diluted*** ....

Thus, the paper of Zong teaches away from using pressure on aggregated proteins, in sharp contrast with the current invention. Zong took care to remove aggregates from samples prior to pressurization. In the present invention, the inventors use aggregates as the starting material.

Furthermore, there is no evidence presented by Zong that the proteins to which high hydrostatic pressure was applied were indeed aggregated. In fact, Zong present data that argues ***against*** the presence of aggregated protein in the samples that they pressurized. Figure 6 shows changes in the absorbance ultraviolet spectra of the recombinant apoCPO constituted with heme during its incubation under high pressure. There is no change in the difference spectra in the wavelength range around 500-550 nm as a function of pressure or temperature. Under the conditions used in Figure 6, Zong found that the apoCPO bound heme to form the holoenzyme. If pressure treatment caused disaggregation, a large change in the difference spectra in this range would have been expected (1). Thus, there is no evidence that aggregated protein was present in the material that Zong treated with high pressure.

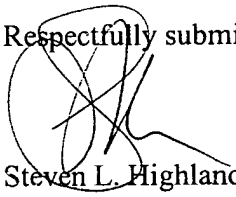
Finally, there is no indication in the paper that Zong used controlled depressurization. The authors clearly define their pressurization protocol. "All the samples were pressurized with gradually increased hydrostatic pressure to 207 MPa in 34.5 MPa increments ...." p. 12421, column 1. In contrast, the depressurization step is minimally described "after pressure release ...." p. 12421, column 2. Again, this teaches away from the present invention, in which the depressurization step is controlled.

In sum, applicants again respectfully submit that the present invention cannot be anticipated by Zong for the simple reasons that (i) Zong did not utilize aggregate as a starting point for refolding, and (ii) Zong did not used stepped depressurization. For either or both of these reason, applicants believe the rejection to be improper, and respectfully request its withdrawal.

IV. Conclusion

In light of the foreging, it is respectfully submitted that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. Should Examiner Guttman have any questions, he is invited to contact the undersigned attorney at (512) 536-3184.

Respectfully submitted,



Steven L. Highlander
Reg. No. 37,642
Attorney for Applicants

FULBRIGHT & JAWORSKI, LLP
600 Congress Avenue, Suite 2400
Austin, Texas 78701
Date: November 30, 2001